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Review

Transmission electron microscopy in molecular structural biology: A historical survey

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Dedication: This article is dedicated to the memory and scientific contribution of the late Robert (Bob) W. Horne, Milan V. Nermut and Marc Adrian, three exceptional hands-on electron microscopists.

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ABSTRACT

In this personal, historic account of macromolecular transmission electron microscopy (TEM), published data from the 1940s through to recent times is surveyed, within the context of the remarkable progress that has been achieved during this time period. The evolution of present day molecular structural biology is described in relation to the associated biological disciplines. The contribution of numerous electron microscope pioneers to the development of the subject is discussed. The principal techniques for TEM specimen preparation, thin sectioning, metal shadowing, negative staining and plunge-freezing (vitrification) of thin aqueous samples are described, with a selection of published images to emphasise the virtues of each method. The development of digital image analysis and 3D reconstruction is described in detail as applied to electron crystallography and reconstructions from helical structures, 2D membrane crystals as well as single particle 3D reconstruction of icosahedral viruses and macromolecules. The on-going development of new software, algorithms and approaches is highlighted before specific examples of the historical progress of the structural biology of proteins and viruses are presented.

Introduction

> Search where we will among created things, far as the microscope will allow the eye to pierce, we find organization everywhere. Large forms resolve themselves into parts, but these parts are but organized out of other parts, down so far as we can see into infinity
>
> [Froude, 1871]

Any historical account is bound be a somewhat blinkered presentation, limited by the information at hand as well as the personal perspective and experience of the author. So, it is perhaps best to acknowledge immediately that of the many scientists who have contributed to this field over the past ~70 years, only relatively few can be mentioned here; indeed there is a vast scientific literature on transmission electron microscopy in structural biology. I apologize to any and all I may have offended unintentionally by omitting comment on their contribution to this discipline. This review will emphasise initially the earliest pioneering contributions, which today are all too readily overlooked. Whilst many today consider that any published data of more than 5 years old is essentially Stone Age and likely to be of little remaining significance, to look into and to evaluate the history of transmission electron microscopy (TEM) in structural biology has, for the author, turned into an extraordinarily interesting task. Indeed, since compiling the introduction to my doctoral thesis: "*The Structure of the Red Blood Cell Membrane Using the Electron Microscope*" in 1968 at the University of Edinburgh, I have not dwelt greatly on historical matters until now. Some inevitable brevity has, sadly, been necessary, since if given full justice this fascinating topic could readily fill a whole book!

Before the digital age it was easy to overlook past publications, and thereby omit even fairly recent literature when compiling a manuscript. Today with the ready availability of on-line literature searches within several scientific databases, there is less or even no excuse for significant omission. However, due to the limited page allocation when preparing this historical review of macromolecular

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electron microscopy inevitably means that the account presented here will be somewhat restricted. Luckily, many of the older journal articles have been digitised by the publishers and are now available in electronic format. It is unfortunate that much material of interest that appeared in older books is not yet available on-line, although this is fast becoming the norm as present-day e-books become widely available. Good academic and institute libraries should, however, still have at least some of the older EM books on their shelves. The international book supplier AbeBooks (www.abebooks.co.uk/www.abebooks.com) has many of these available.

Accepting that: "*If I have seen further it is by standing on the shoulders of giants*" (Isaac Newton), it must be acknowledged that the first TEM was constructed by the electrical engineers Ernst A.F. Ruska and Max Knoll in 1931. More than fifty years' later, Ruska shared the 1986 Nobel Prize for Physics, "*for his fundamental work in electron optics and for the design of the first electron microscope*". The TEM was not used greatly for biological studies until the 1940s, although Ladislaus Laslo Marton (1934) working in Belgium is often credited as being the first to attempt to study biological samples by TEM [1]. Ernst Ruska's brother, Helmut, contributed to the early biomolecular applications in Germany [2,3], as did Manfred von Ardenne [4], studies that were undoubtedly disrupted by the 2nd world war. Through the 1940s in other European countries and in the US, several biological scientists started to use the first commercially-available TEMs, perhaps foremost among these was Ralf W.G Wyckoff [5–13]. Wyckoff used predominantly the metal shadowing approach, but he also studied untreated air-dried biological specimens that revealed little structural detail [14]. It is pertinent that the first attempts to define the size and shape viruses and macromolecules were made early in the 1940s [15]. Francis O. Schmitt and colleagues commenced their series of impressive studies on collagen, primarily using metal shadowing but also with positive staining of thinly spread samples, which firmly established molecular studies on collagen fibril assembly that continued through the next decade [16–18].

During the 1950s the established metal shadowing technique continued strongly and was joined, if not overtaken, by negative staining through the 1960s and 70s, to be complemented from the mid-1980s onwards by the plunge-freezing cryo-technique for the preparation of unstained vitreous specimens. The use of all three approaches for specimen preparation has continued to the present day [19] (see below in the section on techniques).

From the above it is easy to define the start of biomolecular electron microscopic applications, but historically it is less easy to decide the limiting date for the purposes of this review; some link is therefore made to more recent work. The current period will, however, be covered in the Overview of the subject by Richard Henderson [20] and in the subsequent articles in this Special Issue of the *Archives of Biochemistry and Biophysics* devoted to the impact of TEM in structural biology. Since the 1940s the increase in number of applications of the TEM for biomolecular studies through to the present day has been dramatic (Fig. 1), with the more precisely defined discipline of structural biology emerging only in the past ~20 years. Indeed, one has to consider the progressively changing terminology of biological/biomedical academic Departments and research Institutes, ranging from Zoology, Botany, Biological Chemistry, Biochemistry, Biophysics, Bacteriology/Microbiology, Histology/Cytology, moving to Cell Biology, Molecular Biology, Virology, Biomedical Sciences, Biological Crystallography and perhaps most recently and pertinent for the present article, Structural Biology. Although initially the term molecular biology encompassed several of these disciplines, more generally it has come to emphasise nucleic acid-related studies and molecular genetics, which strongly pervades the whole of present day biological and medical research. It is notable, however, that on the whole electron

Frequency of Published Papers: Electron Microscopy and Protein Search in PubMed

1941-2010

1941-1950	7
1951-1960	56
1961-1970	338
1971-1980	1387
1981-1990	1909
1991-2000	1640
2001-2010	2280

Fig. 1. A tabulated survey of the number of publications dealing with electron microscopy and proteins as the search terms, shown per decade from 1941–2010, from a PubMed search. A dramatic increase in number of papers occurred through to 1990, that then levelled out to some extent.

microscopy has not emerged as an academic discipline in its own right as far as, for instance, biological crystallography. Rather, TEM has remained a major research tool, used widely in academic Departments and research Institutes, many of which have included an EM laboratory, but more often in a broadly defined Microscopy or Bioimaging Unit. In addition, several national multi-user core facilities for electron microscopy, including macromolecular imaging, have been established with governmental, industrial and even charitable support. Thus worldwide, biomedical applications of TEM continue to expand, although there are occasional instances of institutional neglect, sometimes due to lack of finance for both equipment and personnel or a general lack of understanding in higher management what a well-resourced EM unit can achieve for perhaps the largest range of samples in structural biology.

The overlap of TEM study of cellular ultrastructure with molecular structure became apparent in the first and second editions of "*Biological Ultrastructure*" by J. Bryan Finean and Arne V. Engström (1958 and 1967). The indispensability of the EM for molecular studies was highlighted in "*The Electron Microscope in Molecular Biology*" by Geoffrey H. Haggis (1966), which also placed more molecular aspects within a cellular context. The Ciba Foundation organized a symposium on the "*Principles of Biomolecular Organization*", published in book format in 1966. Many of the scientists who contributed to this symposium placed a strong emphasis on TEM molecular data. At least two articles within the Cosslett Festschrift (1979), published as an issue of the *Journal of Microscopy*, were of a biomolecular nature, and in 1980 the proceedings of an international workshop on "*Regular 2-D arrays of biomacromolecules: Structure determination and assembly*" sponsored by the DFG appeared as the book "*Electron Microscopy at Molecular Dimensions*", edited by Wolfgang Baumeister and Wolrad Vogell (now available from Springer as an e-book). John Sommerville and Ulrich Scheer in their "*Practical Approach*" book "*Electron Microscopy in Molecular Biology*" (1987) achieved a stronger molecular emphasis. This latter overlapped with a series of six books "*Electron Microscopy of Proteins*" edited by J.R. Harris and R.W. Horne (1981–1987), which dwelt further on truly macromolecular aspects, mostly prior to the introduction of cryo-electron microscopy. Strangely and, in hindsight, a strong proposal for a further volume on the then emerging field of cryo-electron microscopy was not accepted by Academic Press! This was instrumental in the commencement of the Pergamon

Press review journal *Electron Microscopy Reviews: Subcellular and Biomolecular Structure* (1988), which subsequently merged with *Micron and Microscopica Acta* as the review section the Elsevier journal *Micron* in 1993. In retrospect, it is indeed regrettable that this merger occurred, as the main thrust of the review journal, firmly indicated by the sub-title, was then lost.

Virus structure has received repeated attention in single- and multi-author book format since the earlier days of biological TEM. One of the first, entitled "*Ultrastructure of Animal Viruses and Bacteriophages*" edited by Albert J. Dalton and Francoise Haguenau (1973), provided a strong cellular link through to the study of isolated viruses. With other virology books, Dalton and Haguenau's volume led to the increasing structural emphasis on the wide range of virus families [21–25]. These books provided in-depth surveys of the subject, with a clear historical progression, along with numerous articles in review journals [26] and series such as *Advances in Virus Research*, extending from Vol. 1 (1953) [27]. Such publications all serve to emphasise the important role of TEM in virus research, within the context of the historical advancement of structural biology.

Without unduly labouring the matter of terminology, it is clear that present-day molecular structural biology split off from the more tissue and cell-based ultrastructure research, as can be seen from the evolving title of the *Journal Ultrastructure Research* to the *Journal of Ultrastructure and Molecular Structure Research*, then in 1990 to the *Journal of Structural Biology*. The *Journal of Biophysical and Biochemical Cytology* moved the other direction becoming the *Journal of Cell Biology*. However, with the commencement of the *Journal of Molecular Biology* in 1959, a natural home for many TEM-based structural studies was immediately established. The existing biochemical and virological journals, eg *Biochimica et Biophysica Acta* and the *Journal of Virology* continued to play an important role alongside the mainstream journals such as *Nature, Science, Biochemistry* and the *Proceedings of the National Academy of Sciences* (*PNAS*) that also received a good complement of early macromolecular TEM manuscripts, journals that have been joined in more recent years by the likes of the *EMBO Journal* and *Molecular Cell*. The microscopy-specific journals, *Ultramicroscopy*, the *Journal of Microscopy* and *Micron*, have also received a fair complement of molecular manuscripts. Over the years the Royal Society has held several discussion meetings on virology, new developments in electron microscopy and on biological membranes and envelopes, all of which were published as issues of the *Philosophical Transactions of the Royal Society of London*.

Although, as indicated above, a firm publishing distinction was not initially made between cellular ultrastructure, particulate protein structure and virus structure, the three often appear alongside in books and in journals. Progressively and more recently, the biochemical journals have tended to include molecular structure articles and the cell biology journals the more ultrastructural articles, but the two areas have tended to overlap through to the present day within the *Journal of Structural Biology*, whereas the *Journal of Molecular Biology*, *Nature Structural Molecular Biology*, *Structure* and *Current Opinion in Structural Biology* tend to contain only the more molecular articles. Over the years the *Journal of Structural Biology* and *Micron* have published impressive special issues covering aspects of macromolecular structure, and *Annual Reviews*, *Advances in Protein Chemistry and Structural Biology* and other book series have included reviews on molecular electron microscopy. These various publishing examples have depicted the historical progression of the expanding field of macromolecular electron microscopy. Although scanning transmission electron microscopy (STEM) has made a useful contribution to macromolecular mass analysis [28], but in the present context the contribution of STEM is considerably less than that from TEM, and will not be expanded upon here.

The biochemical techniques of subcellular fractionation, protein and virus purification, are the foundations to much of what has been studied by TEM and other structural biology techniques (e.g., *Biochemical Society Symposia No. 23*, "Methods of Separation of Subcellular Structural Components", 1963). The TEM contribution of Albert Claude in this area was succinctly reviewed by George Palade in 1971 [29]. Briefly, it was the development of differential centrifugation followed by sucrose, ficoll and caesium chloride gradient centrifugation, together with gel filtration and ion exchange chromatography, and more recently zinc chelate and other types of affinity chromatography (predominantly for recombinant material) that has facilitated this progress. Isolated bacterial cell walls and ribosomes, eukaryotic mitochondria, chloroplasts, membrane fractions and numerous purified soluble and fibrillar cytoskeletal proteins were studied in the early days of TEM, together with extracellular matrix fibrillar proteins such as collagen, elastin, amyloid and even cellulose fibrils [13,30–32]. The development of the biochemical purification techniques was essential for progress made during the early and subsequent TEM study of virus particles, together with their protein and nucleoprotein components.

The importance of the development of low temperature techniques for the TEM study of biological samples, isolated macromolecules and viruses also cannot be overstated. Appreciation that the maintenance of hydration by rapid freezing (even freeze drying) would be of structural benefit to the sample was indicated in 1960 by Vol. 85 of the *Annals of the New York Academy of Sciences*, which included an article by Humberto Fernández-Morán [33] on rapid freezing in liquid helium. The contribution of Robert M. Glaeser and his colleague Kenneth A. Taylor in connection with low temperature electron crystallography through the 1970s must also be mentioned [34,35], together with the more recent contribution of Kenneth H. Downing. However, the ability to produce vitrification of water by rapid freezing rather than crystallization, was crucial to the further development of this method. This was achieved by using liquid nitrogen or more efficiently by using liquid nitrogen-cooled liquid propane or ethane to avoid the reduced cooling-rate of microbubbles when plunging directly into liquid nitrogen, a technique developed by Jacques Dubochet and his colleagues in the early 1980s (see below, in section on unstained vitrified cryo-specimens). Much has been written subsequently on cryo-electron microscopy to indicate the historical development of this technology [36–40].

The overall aim of this historical review is to place emphasis upon the contribution of the TEM to macromolecular aspects of structural biology, with the understanding of protein structure foremost, primarily relating to size and shape of macromolecules, macromolecular assemblies and viruses, leading to digital 3D reconstructions at progressively higher resolution. This review will be illustrated by discussion of techniques and published studies of animal, plant and bacterial viruses, cellular and extracellular protein molecules, nucleic acids and nucleoproteins, carbohydrates, lipids, lipoproteins and biological membranes.

Specimen preparation techniques for TEM and their application

The various techniques/protocols for preparing biological and more specifically biomolecular specimens for TEM study have from the 1950s through to the present day been documented thoroughly within numerous multi-author and single author texts (e.g., *Introduction to Electron Microscopy* by Cecil E. Hall (1953, 1966, 1983); *Electron Microscopy: Technique and Applications,* Ralph Wyckoff (1949); *Techniques for Electron Microscopy*, edited by Desmond Kay (1961 and 1965); *Practical Methods in Electron Microscopy*, 12 volumes, series editor Audrey M. Glauert (1972–1999);

Cryotechniques in Biological Electron Microscopy, edited by Rudolf Alexander Steinbrecht and Karl Zierold (1987); *Negative Staining and Cryoelectron Microscopy* by J. Robin Harris (1997); three editions of *Electron Microscopy: Methods and Protocols*, edited by M.A. Nasser Hajibagheri (1999) and John Kuo (2007, 2014); *Electron Crystallography of Biological Macromolecules*, edited by Robert M. Glaeser, David De Rosier, Wah Chiu and Joachim Frank (2007); *Electron Crystallography of Soluble and Membrane Proteins* edited by Ingeborg Schmidt-Krey and Yifan Cheng (2013). The value of these texts, and others, should not be underestimated as they contain a wealth of material documenting the increasing body of technical knowledge that underlies the historical development of biomolecular electron microscopy. In addition, most techniques have also appeared within the scientific journals and are readily accessible in libraries and on-line.

(Ultra)thin sectioning

Thin sectioning of resin/plastic-embedded specimens for TEM developed from the early 1940s, extending from light microscope microtomy, the technique generally being termed ultramicrotomy. Thus, the manufacture, ready availability of ultramicrotomes, using glass and later diamond knives, was essential for the function of all EM laboratories. Along with thin sectioning, an understanding of tissue fixation and pre-embedding staining, dehydration, resin permeation and polymerization, and thin section post-embedding staining were all necessary. Many scientists contributed to this technical development through the 1940s onwards, notably Thomas F. Anderson, Audrey M. Glauert, Edward Kellenberger, George Palade, Walther Stoeckenius, Fritof S. Sjöstrand, Keith R. Porter and J. David Robertson, to mention but a few.

Apart from showing detail of tissue and cellular ultrastructure, thin section TEM also revealed the presence of viral particles (including intracellular viral crystals), fibrillar proteins such as collagen, skeletal muscle actomyosin, keratin and intermediate filaments, amyloid fibrils and chromatin. Crystalline microbodies and protein occlusions within cells were also readily revealed by thin sectioning. At the particulate level, glycogen, ribosomes and large protein molecules such as ferritin (revealed by the presence of its iron hydroxide core), the high molecular mass molluscan hemocyanins (Mr ~8 MDa and above) and the annelid hemoglobins (erythrocruorins) (Mr ~3.5 MDa) [41] were also detected by thin sectioning. Within membrane structures, molecular order was initially indicated, such as within mitochondrial cristae, the nuclear envelope, plasma membrane gap junctions, bacterial, algal and fungal cell walls, and the bladder luminal membrane. Molecular detail of all the above structures, and others, was subsequently confirmed and expanded by the use of the additional specimen preparation techniques (see below).

Extracellular collagen fibrils and fibril bundles within tissues were readily defined by thin sectioning due to their regular width and linear banding [42], an indication of the precise molecular overlap and spacing of the collagen heterotrimers as a *quasi*-crystalline cluster within individual fibrils (see also below, metal shadowing). Similarly, the myofibril array within skeletal muscle cells was initially defined by thin sectioning, contributing greatly to the sliding filament theory for muscle contraction developed by Hugh Huxley and Jean Hanson [43–45]. In addition the study of nerve myelin, chloroplast grana, retinal rod outer segment discs, bacterial cell walls and other membrane systems benefited greatly from thin sectioning, together with neuromuscular and synaptic junctions and plasma membrane cell–cell junctions, in many cases supplying an ultrastructural foundation for subsequent macromolecular and functional investigations. Thin sectioned vertebrate yolk-platelet crystals were studied in detail by Rainer H. Lange [46], although Walter Hoppe in 1968 showed the limitation of plastic-embedded protein crystals for electron and X-ray diffraction [47].

Metal shadowing

The metal shadowing and replica techniques (shadow casting) became available during the early biological TEM study of samples isolated from tissues and cells, primarily due to the existing high vacuum technology and the ability to evaporate carbon and metals such as chromium, gold, platinum, palladium or tungsten at an oblique angle onto a mica surface upon which particulate matter had been thinly spread, thereby creating a "shadow-like" coated image of the material under study. This approach was incorporated into the freeze-fracture technique, which has also made a considerable contribution to cellular ultrastructure. David E. Bradley was a leading contributor to this shadowing technology, along with Cecil E. Hall who revealed the linear beaded flexible nature of the acid-soluble collagen molecule (heterotrimer) [48,49]. Molecular detail revealed by the metal shadowing technique was dependent upon the absence of salt and buffer when samples were thinly spread and dried, but the removal *in vacuo* of volatile components such as glycerol, acetic acid and ammonium acetate or carbonate prior to shadowing was an extremely useful feature of this technology, in particular for elongated molecules such as fibrinogen, myosin, spectrin and actin fibres, in addition to collagen.

Metal shadowing, single angle and rotary, became an established specimen preparation technique for the study of virus structure, fibrils and macromolecular assemblies through the 1950s to 1980s, with Ralph W.G. Wyckoff making a significant macromolecular contribution [50], but the use of metal shadowing has declined somewhat in more recent years as sample preparation, instrument and methodologies have improved and developed. Since the early days of biological EM, small protein crystals were also studied by metal shadowing [9,11]. Several of the contributions within the "Practical Approach" book "*Electron Microscopy in Molecular Biology*" (edited by John Summerville and Ulrich Scheer, 1987) show a broad range of shadowing applications, stemming from the DNA and chromatin spreading techniques extending from the approach initially developed by Albrecht Kleinschmidt in 1959 [51]. Exponents of metal shadowing for the study of isolated bacterial cell wall macromolecules and viruses, such as Thomas F. Anderson, Henry Slayter, Kurt Mülethaler, Francis O. Schmitt, Heinz Gross, Milan V. Nermut, John E. Heuser and Uwe Sleytr, and others, all published meaningful studies.

Heinz Gross [52], Henry Slayter [53] and George C. Ruben [54] in particular pushed the metal shadowing technique towards its resolution limit, and interest has been shown in the site-specific metal cluster decoration of protein molecules [55]. Metal shadowing examples are shown for platinum carbon shadowing of the *n*-butanol protein extract prepared from human erythrocyte membranes is shown in Fig. 2 [56], and of experimentally re-associated keyhole limpet hemocyanin type 2 (KLH2)[1] helical tubules following freeze fracture (Fig. 3) (courtesy of the late Milan V. Nermut) [57]. John E. Heuser developed a freeze-drying and deep etching procedure that showed considerable potential at the macromolecular level [58]. Metal shadowing was used to demonstrate the presence of the ordered array of bacteriorhodopsin molecules within the light harvesting purple membrane of *Halobacterium salinarum* (Fig. 4) [59]. This, and other shadowing data, was of considerable significance for the subsequent development of the study of bacterial S-layers and a wide range of 2D membrane crystals (see below in section on Electron crystallography).

[1] *Abbreviations used:* KLH2, keyhole limpet hemocyanin type 2; Prx-II, peroxiredoxin-II; LaB_6, lanthanum hexaboride; scFv, small chain variable; X-FELs, X-ray free electron lasers.

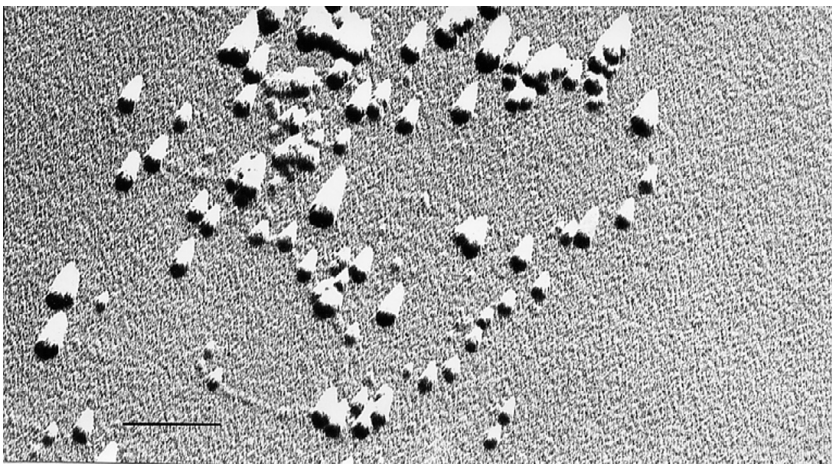

Fig. 2. Single angle platinum shadowed protein particles present in the *n*-butanol extract from human erythrocyte ghosts. The scale bar indicates 200 nm. (JRH, Ph.D. Thesis, Fig. 30, University of Edinburgh, 1968) [56].

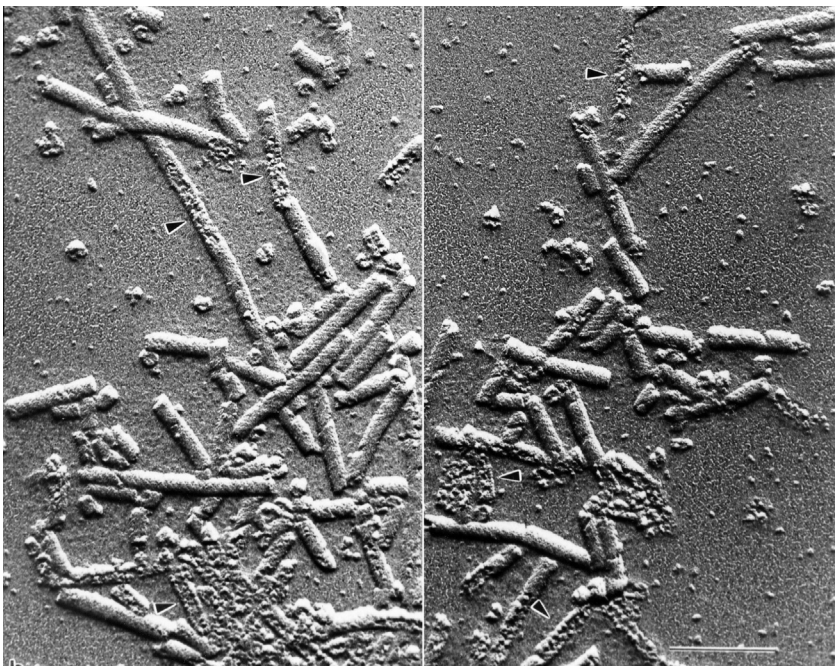

Fig. 3. Freeze-fractured and metal shadowed tubules and multidecamers of keyhole limpet type 2 (KLH2), from subunit monomer reassociated in the presence of 0.1 M calcium chloride and 0.1 M magnesium chloride. Note the helical pattern on the tubule and multidecamer surface. Arrowheads indicate fractured tubules. Specimen preparation and image courtesy of the late Milan V. Nermut. Modified from Harris et al. (1997) [57], with permission of Elsevier.

Although its use has declined in recent years, metal shadowing continues to be useful for the study of macromolecules and other biological samples [60], emphasising the importance of this specimen preparation technique for macromolecular studies, used from the earliest days of TEM.

Negative staining and cryo-negative staining

The principle of negative staining is rather old, having been used in bright field light microscopy to reveal thinly spread particulate material such as bacteria, spores, protozoa, parasites and oocysts immersed in a black or dark-coloured dye solution. The microscopical image thereby appears light within the dark or coloured surrounding background of stain. For TEM the same principle applies, in this case with the biological particles surrounded and permeated by initially an aqueous and then a dried amorphous layer of heavy metal-containing stain.

Whilst examples of spurious/anomalous TEM negative staining can found in the literature [61,62] it was Sydney Brenner and Robert W. Horne in 1959 [63] who established a working procedure for the negative staining of viruses, using tobacco mosaic virus and turnip yellow mosaic virus; the technique was then rapidly utilized for the study of many other plant, animal and bacterial viruses [64] (Fig. 5). Phosphotungstic acid had been used for some time as a positive stain for cellular and viral material and it was found that with inadequate washing of samples spread on carbon support films residual stain surrounded the particles, a reverse-contrast image could be generated. Once this was appreciated, neutral pH phosphotungstate solution was used, with the firm intent of leaving a thin spread of aqueous stain that surrounded the biological material and permeated any available aqueous channels within

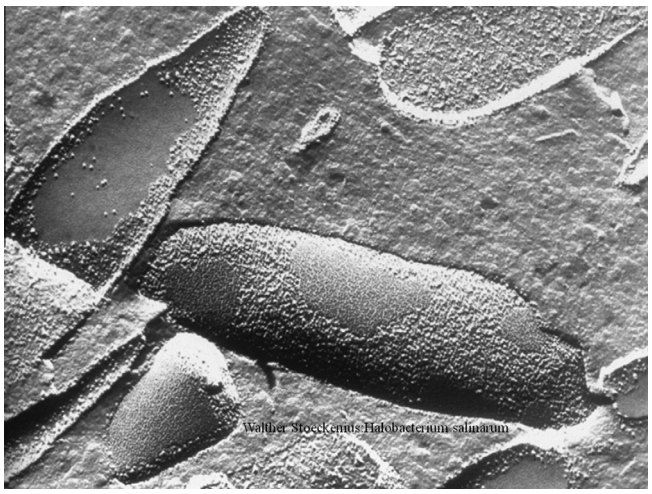

Fig. 4. A freeze-etch preparation of *Halobacterium salinarum* (*halobium*) showing discrete patches of purple membrane, subsequently used for Unwin and Henderson for 2D crystal analysis. Equivalent to Fig. 11 of Blaurock et al. (1976) [59]. Micrograph courtesy of Richard Henderson, produced by Walther Stoekenius.

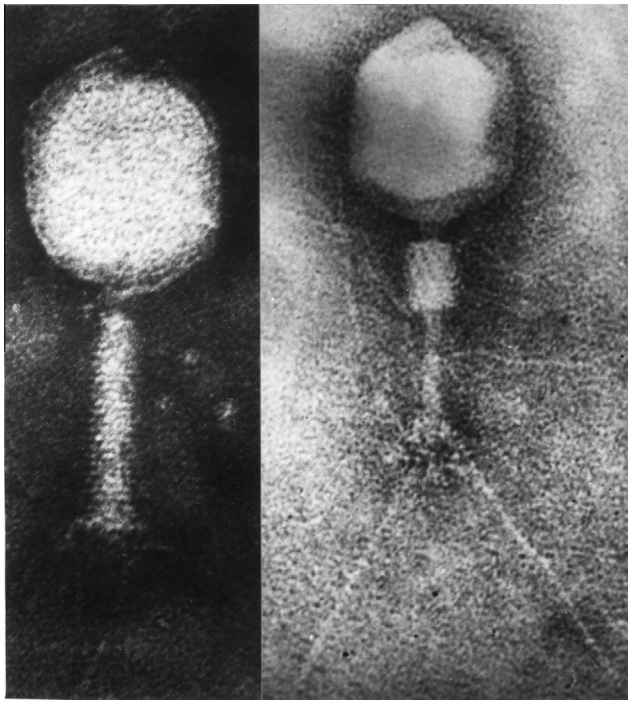

Fig. 5. Bacteriophage T2 particles negatively stained with phosphotungstate showing the periodic structure of the tail (A) with peroxide-triggered contraction of the tail sheath revealing the inner core (B). Micrograph courtesy of the late Robert W. Horne. From Horne and Wildy (1974) [64], with the permission of Elsevier.

the sample. Following air-drying, viral and other particulate samples were essentially embedded in a thin layer of amorphous stain. Uranyl acetate also came into use as negative stain, followed by sodium silicotungstate, ammonium molybdate, and to a lesser extent methylamine tungstate and methylamine vanadate. Low contrast negative staining has also been performed using salts of low atomic weight metals, the crucial feature being that in all cases the thin layer of dried negative staining salt should be amorphous.

In the initial work viral samples were usually mixed with the negative stain solution and sprayed as droplets onto a carbon support film, and this was successfully applied to protein molecules by Robin C. Valentine [65]. Deposition and spreading of sample and stain droplets direct to carbon support films was also introduced by Hugh E. Huxley and Geoffrey Zubay in 1960 [66], and independently by David E. Bradley during his extensive studies on bacterial pili, bacteriophages and bacteriocins [67]. The author has followed this approach, having seen it demonstrated during a visit to the laboratory of Ennio-Lucio Benedetti in Amsterdam in 1965, where he was shown the "Parafilm droplet" procedure for negative staining [68], which with minor variations he has used to the present day [69,70]. It is clearly apparent that the rapid development of the fields of animal and plant and bacterial virology benefited greatly from the negative staining technique [71,72]. An early assessment of negative staining was by Valentine and Horne in 1962 [73], in which they outlined the then *state of the art* technique for studying biological *particles*. This paper is of some historic interest because these two individuals were significant and serious scientific competitors; how many of today's key researchers would co-publish a key methods development paper with their closest rival? A survey of the application of negative staining to the study of enzymes was published in 1974 by Rudy Haschemeyer and Etienne de Harven [74].

It is pertinent to consider further the historical development of negative staining in relation to the use of carbon support films. Several historical variant negative staining approaches have been published, all of which have offered some benefit in the hands of those who developed the modifications. In the carbon floatation procedure advanced by Robin Valentine and colleagues [75], a carbon film was floated off a mica surface directly onto a sample solution containing glutamine synthetase. In the negative staining–carbon film (NS–CF) procedure, developed by Robert W. Horne and Ivonne Pasqualli-Ronchetti [76], the sample was mixed with negative stain (ammonium molybdate, also with polyethylene glycol [77]), spread on mica, air-dried and coated with carbon; the carbon film + adsorbed virus or protein was then floated off onto a second negative stain solution (usually uranyl acetate) and placed onto an EM grid, with excess stain removal (Fig. 6). A major drawback of this NS–CF procedure is that the freshly deposited carbon plus adsorbed sample does tend to generate shallow/partial depth negative staining, but an advantage is that 2D crystalline arrays can be produced, a benefit that has not yet been fully

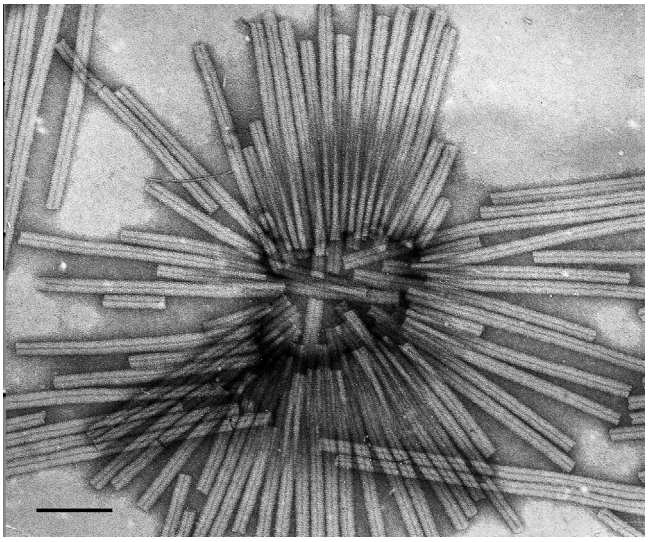

Fig. 6. Negatively stained TEM image of tobacco mosaic virus (TMV). The specimen was prepared by the late Robert W. Horne using the negative staining-carbon film procedure. From: Electron Microscopy in Biology, A Practical Approach (1991) p. 224, with the permission of Oxford University Press.

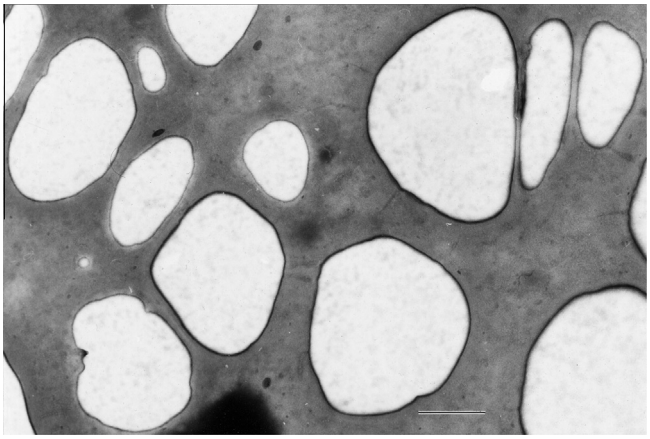

Fig. 7. A representative low magnification TEM image of the thin dried film of Human erythrocyte Prx-2 sample + ammonium molybdate, trehalose and PEG, to show even thin spreading across the holes of a holey carbon support film, essentially similar to unstained vitreous cryo-specimens. The scale bar indicates 1 μm. Image from Meissner et al., Micron 38 (2007) 29–39 [87], with the permission of Elsevier.

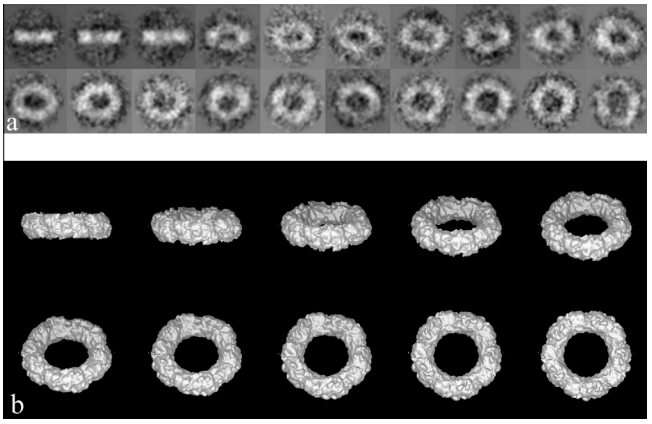

Fig. 9. (a) An orientation sequence of 20 class averages of human erythrocyte Prx-II projection images (external diameter approx. 130 Å), from vertical through to horizontal orientation, produced within IMAGIC-5 from 885 single images of negatively stained molecules, equivalent to those shown in Fig. 8. (b) A continuous stereo sequence of the surface-rendered 3-D reconstruction of Prx-II. Reprinted from Harris et al., Biochim. Biophys. Acta 1457 (2001) 221–234, with permission of Elsevier [82].

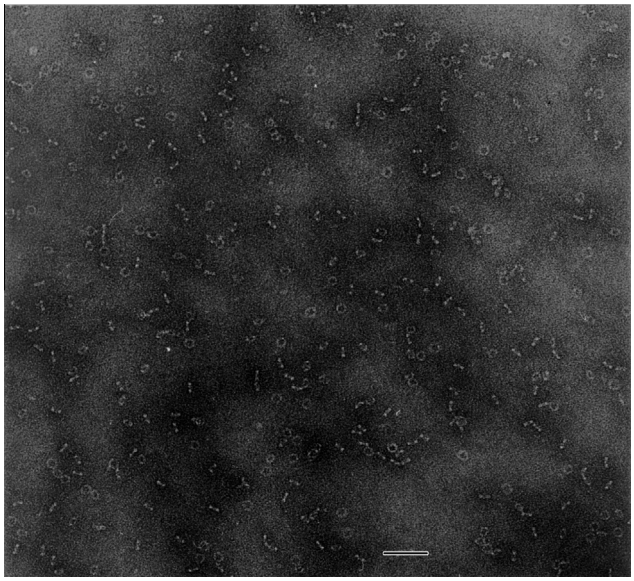

Fig. 8. Human erythrocyte Prx-II molecules spread in the presence of 5% ammonium molybdate-0.1% trehalose (pH 6.9) across small holes of a holey carbon support film. Note the widely varying images due to the broad range of angular orientations of the Prx-II molecules embedded within the thin film of negative stain. The scale bar indicates 100 nm. Reprinted from Harris et al., Biochim. Biophys. Acta 1457 (2001) 221–234, with permission of Elsevier [82].

exploited at the macromolecular level (see below for comparison with negative staining across holes).

A distinction can thus be made between the prior mixing of sample and negative stain before application to the carbon support film, where sample-stain interaction can occur, and the initial prior adsorption of sample to a carbon surface followed with removal of salt, buffer and other solutes by water washing, and then negative staining. In both cases, the depth of negative stain surrounding/embedding the biological particles can be variable, resulting in incomplete to total coverage, which is expressed as variability of the electron image. To overcome this variation, the carbon sandwich negative staining procedure was developed [78], but this carries the risk of particle flattening.

An alternative that avoids both incomplete embedding in stain and molecular flattening was advanced by Hugh E. Huxley in one of the earliest publications using negative staining [79]. Here, the particulate sample and negative stain is spread across small holes in a perforated or holey carbon support film or across the corners of a broken carbon film. This holey carbon negative staining technique was established as a routine procedure many years later using an ammonium molybdate-trehalose mixture, which assisted the spreading of sample and stain across the holes (Fig. 7) [80,81]. An example of this approach was the imaging and 3D reconstruction of the ~240 kDa decameric human erythrocyte peroxiredoxin-II (Prx-II) [82], as shown in Fig. 8. Three-dimensional reconstruction of this molecule in IMAGIC from negative stain images (ammonium molybdate-trehalose) created a molecular envelope, at a TEM resolution of 1.9 nm in this instance (Fig. 9), essentially equivalent to the solvent excluded molecular surface from X-ray crystallography. For historical comparison, the first negative stain TEM analysis of this protein in 1969 (then termed Torin) is shown in Fig. 10 [83]. Here the ten-fold rotational symmetry was revealed, but the fact there are in reality five domain-swapped subunit dimers present (Fig. 9) was not revealed by the Markham photographic rotational enhancement technique used at this time.

Furthermore, it is of considerable significance that surface forces at the fluid-air interface during negative staining across holes, as in the NS–CF procedure, can potentiate the formation of 2D crystals, in this instance supported *only* by the thin film of stain + trehalose + PEG or indeed trehalose + PEG alone. Continuing this historical development of negative staining to the present day, Zhang and colleagues [84] have successfully used an *optimized* protocol for negative staining of lipoproteins and cholesteryl ester transfer protein.

A direct link between the air-drying holey carbon negative staining procedure and the cryo-negative staining procedure established by Adrian et al. is apparent [85], which in turn extended from the established plunge freezing technique used for the preparation of unstained vitreous specimens [86]. Apart from its usefulness for imaging single particles, this cryo-negative staining procedure also possesses the ability to induce 2D viral and protein crystal formation, again enhanced by the presence of PEG (Fig. 11). Higher order molecular macromolecular assemblies can also be produced [87]. An alternative carbon sandwich cryo-negative staining procedure has been developed recently by Holger Stark and his colleagues [88].

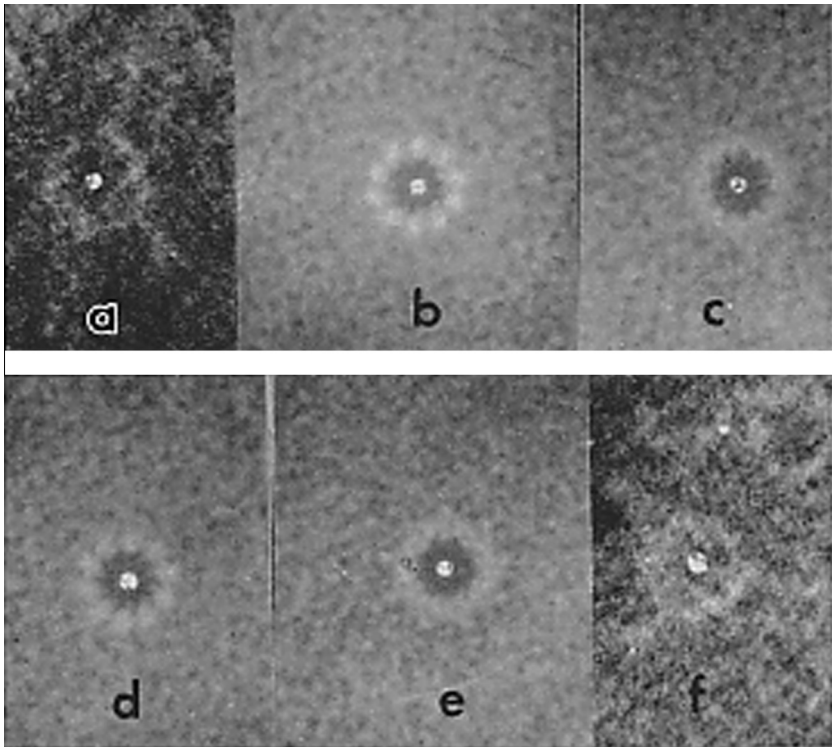

Fig. 10. The Markham photographic rotation technique for contrast enhancement applied to the human erythrocyte torin (subsequently termed Peroxiredoxin-II) orientated in a horizontal plane. Images (a) and (f) have not been rotated. Images (b) and (d) have been rotated about their central point by steps of 360/10 degrees; image (c) by steps of 36/11 degrees and image (e) by steps of 360/9 degrees. The structural interpretation of this analysis is that the ring-like molecule contains ten subunits. From Harris (1969) [83] with the permission of Elsevier.

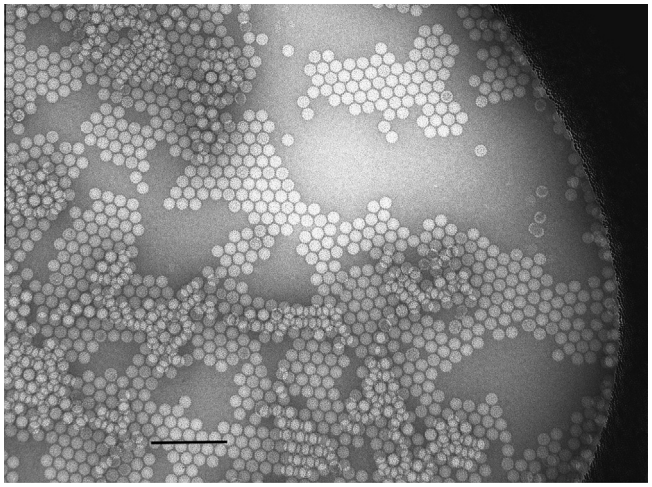

Fig. 11. Brome mosaic virus prepared by cryo-negative staining with 16% ammonium molybdate in the presence of 2% PEG (courtesy of the late Marc Adrian). Note the 2D crystalline arrays. From Harris and De Carlo [70] with the permission of Springer.

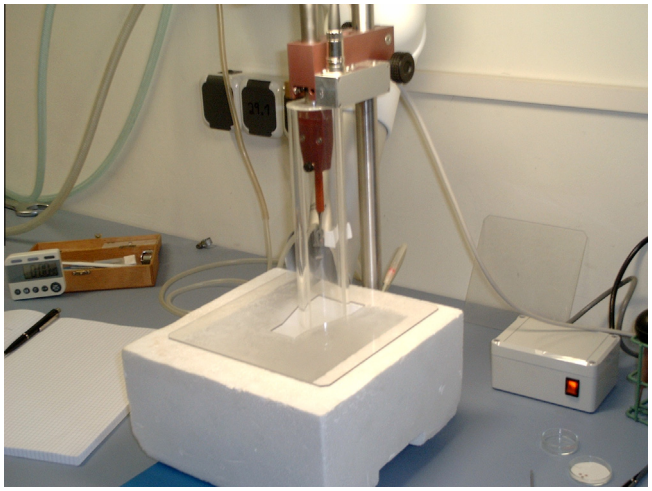

Fig. 12. An example of an early manual plunge-freezing apparatus (*guillotine*) for specimen vitrification, designed and produced by the LMB in Heidelberg, with polystyrene container for liquid nitrogen and a Plexiglas environmental chamber.

Unstained vitrified cryo-specimens

The broad term "cryo-electron microscopy" incorporates many technical approaches, stemming from freeze-drying of samples, freeze-fracture and deep etching, cryo-ultramicrotomy of vitreous sections, slam-freezing, high-pressure freezing and freeze-substitution, etc. Here, I am concerned only with the history, preparation and study of thin-spread unstained vitreous specimens, produced by the plunge-freezing technique. This technique was developed by Jacques Dubochet and his colleagues, in particular by Marc Adrian and Jean Lepault during the early 1980s, at the EMBL in Heidelberg [86]; the topic was impressively reviewed in 1988 [39]. Laboratories around the world rapidly adopted this "*Adrian*" technique, initially with a simple plunge freezing "*guillotine*" (Fig. 12), and more recently with progressively superior commercially available plunge-freezing equipment. The associated technology of cryo-transfer developed in parallel with plunge freezing, in particular the hands of Gatan, Inc. and Oxford Instruments. Successful cryo-electron microscopy of biological material depended upon the development of cryo-specimen holders (Fig. 13) and

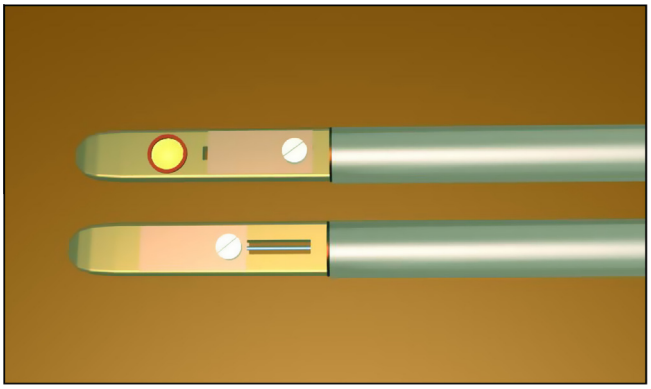

Fig. 13. A diagrammatic representation of the tip of a cryo-specimen holder, showing the grid location and sliding shield open (top), and with sliding shield closed (bottom), for transfer to the TEM airlock. Image courtesy of the late Marc Adrian.

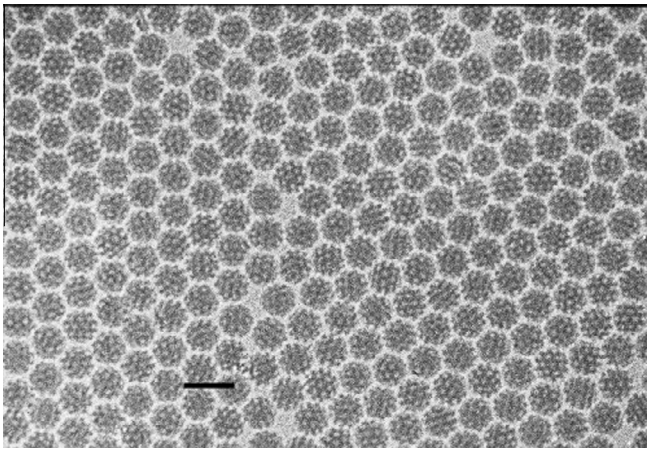

Fig. 14. TEM low-dose image of a two-dimensional array of unstained vitrified Semliki Forest virus (SFV). Note the close-packed 2D array, with virus particles in several different orientations (i.e., in the absence of PEG this is not a 2D crystalline array). The scale bar indicates 100 nm. Micrograph courtesy of Jacques Dubochet, from reference [69] Figure 8.18, with the permission of Taylor and Francis.

cryo-specimen stages that maintained a low specimen temperature within the electron microscope (below 110 K) with an efficient high vacuum system and liquid nitrogen-cooled anti-contamination, along with lens systems that produced adequate contrast from unstained biological material. All these technical improvements have been developed and produced by instrument manufacturers. These developments occurred in parallel with the move from the thermionic tungsten wire filament electron source and lanthanum hexaboride (LaB_6) cathodes (which possess a lower energy spread), to field emission guns that emit a monochromatic (coherent) electron beam, operating at 200 and 300 kV. Low electron dose TEM is an absolute requirement for frozen-hydrated specimens to avoid radiation damage (Fig. 14) [69,86], with appropriate imaging recording systems being incorporated by the instrument manufacturers.

As with negative staining, the nature of the support films used for the preparation of vitrified/frozen-hydrated cryo-specimens has also been the focus of experimentation. Adrian et al. [86] used bare EM grids, but holey (perforated) carbon support films soon became the support of choice because samples were found to spread more evenly across the holes than with bare EM grids. After blotting, the thin unsupported aqueous layers which form following plunge-freezing become stable vitrified films, that could be stored in liquid nitrogen if desired, and subsequently studied at low temperature within the TEM. Today, rather than lab-produced holey carbon films, the regularly spaced size-defined holes on Quantifoil® holey carbon films, produced by semiconductor lithographic techniques, have become the norm. The plunge freezing technique has also been utilized extensively for the preparation of specimen grids containing carbon-supported 2D membrane crystals, which have been prepared when embedded in glucose or trehalose (air-dried and plunge frozen) as well as in vitreous water.

Descriptions of the plunge freezing technique have been presented in protocol format by several authors e.g., [69,89–90] and are available in numerous journal articles e.g., [91,92].

Site-specific labelling of macromolecules

Colloidal gold immunolabelling has long been used as a method for protein localization within tissues and cells, and its application to intracellular and extracellular labelling has been extensive; the technique has continued strongly through to the present day. For isolated macromolecules, immunolabelling with colloidal gold-conjugated IgG molecules, Fab or small chain variable (scFv) antibody fragments has been useful. The antibody alone can be detected as a projection on a molecular surface or can reveal a linkage pattern for molecules, in the case of IgG [93]. Through the 1960s and 70s James A. Lake and his colleagues devoted much effort to the immunolabelling of ribosome particles [78,94] directed towards subunit localization. This group changed their research direction after the 1980s to the field of rRNA sequencing, becoming eminent in the field of molecular genomics and evolution, rather than the further TEM of ribosomes! The strong affinity between streptavidin and biotin has also been utilized for molecular labelling studies. Biotinylated proteins or nucleic acids can be labelled with streptavidin-gold conjugates [95]. The availability of nanogold and undecagold particles extended the possibility for increased precision of molecular labelling, due to the efforts of James F. Hainfeld and his colleagues [96].

Digital image analysis and 3D reconstruction

Background

Although the human brain excels at image interpretation, because of excellent vision, the accuracy of subsequent molecular diagrams and 3D models is inevitably limited. Nevertheless, it is all too easy to dismiss the immediate value of direct visual interpretation of TEM 2D projection images of protein molecules and viruses, which abound in the literature, in favour of computer-generated 2D molecular projection averages from 2D crystals together with 3D reconstructions from 2D crystal tilt series, helical viral particles and fibril 3D reconstruction, along with 3D reconstructions from single viruses and macromolecules.

Image reconstruction from icosahedral viruses developed initially [97,98], followed by protein projection image class averaging from different particle orientations, angular reconstitution and 3D reconstruction, described fully by Joachim Frank in his book "*Three-Dimensional Electron Microscopy of Macromolecular Assemblies: Visualization of Biological Molecules in their Native State*", a topic also reviewed by Marin van Heel and colleagues [99]. The early electron crystallographic averaging provided a broad base from which single particle averaging has greatly extended in recent years, from negatively stained images and more importantly from unstained cryo-images. Digital image analysis and image processing contributed to the subject in an analytical manner by removing the subjective aspects of molecular image interpretation, moving

progressively to higher resolution 3D reconstructions. This was emphasised recently by the publication of three volumes of *Methods in Enzymology* (Vols. 481–483, 2010) devoted to many aspects of this subject, along with two volumes of *Advances in Protein Chemistry and Structural Biology* (Vol. 81, 2010 and Vol. 82, 2011).

Application of Fourier optical [100] and then Fourier digital analysis techniques, extended from the available 3D crystallographic X-ray analysis procedures for the study of protein crystals established by John C. Kendrew, Max F. Perutz, Francis H.C. Crick and others in the Cambridge MRC Laboratory of Molecular Biology through the 1940s and 1950s. These techniques were utilized for the Fourier synthesis of helical and icosahedral viruses [101,102], and then to thin catalase crystals and 2D crystals from bacterial membranes [103,104], work initiated by Aaron Klug (The Nobel Prize in Chemistry 1982 was awarded to Aaron Klug "***for his development of crystallographic electron microscopy and his structural elucidation of biologically important nucleic acid-protein complexes***"). It is worthy of comment that several of Aaron Klug's co-workers have, over a considerable time period up to the present day, made significant contributions in the field of biomolecular electron microscopy, in particular David DeRosier, Donald Caspar, Nigel Unwin, Richard Henderson, Anthony Crowther, Linda Amos and John Finch.

From TEM images recorded originally onto glass photographic plates and subsequently onto celluloid sheet film (e.g., Kodak, Agfa, Ilford), by inserting the photographic film into an optical diffractometer it was possible to generate diffraction patterns that contained averaged information from periodic (initially helical) structures and 2D viral arrays/crystals [105–107]. Thus the diffraction pattern could be indexed and an optically filtered reconstructed image could be produced, a procedure generally termed Fourier analysis. This approach was used successfully for a number of years, through the 1960 and 1970s, until the advent of digital image analysis and the production of computer generated Fast Fourier Transforms from digitally scanned photographic images containing ordered helical or 2D crystalline structures [100]. Due to high resolution limitations, macromolecular image recording by CCD cameras has not taken over from photographic film, but advanced electronic detectors are already beginning to make an impact.

Fig. 15. The first 3D reconstruction by electron crystallography from a 2-dimensional membrane protein crystal of bacteriorhodopsin, at 7 Å resolution, produced by Henderson and Unwin in 1975 [103]. The top and bottom of the model represent the region of the protein in contact with the surrounding solution, the central region containing seven closely packed alpha-helices being surrounded by the membrane lipid. Image courtesy of Richard Henderson, republished with permission from MacMillan Journals.

Electron crystallography

Electron crystallography was first performed within the TEM using low-angle electron diffraction analysis of thin 3D crystalline proteins or microcrystals [35–38,108–111]. Frozen-hydrated collagen fibrils were also studied by electron diffraction [112]. Significant progress was made by Nigel Unwin and Richard Henderson in the early 1970s, prior to the introduction of cryo-technology, when they studied thin catalase crystals and glucose-embedded 2D membrane purple membrane crystals from *Halobacterium halobium* (Fig. 15) [103,104]. From the electron crystallography of 2D planar and tubular (helical) membrane crystals, initially using negative staining then glucose and trehalose embedding and rapid freezing of unstained vitreous specimens [113–116], the combination of electron image and electron diffraction data has proved to be enormously successful. The same cannot be said for electron diffraction analysis of thin or small 3D protein crystals that due to a range of technical and computing limitations, has not yielded high resolution 3D reconstructions until recently [117]. Application of electron diffraction to 2D and 3D crystals of viruses has been limited because of the TEM camera length available for electron diffraction [118]. Modern instruments have undoubtedly improved this technique, but electron diffraction has not yet produced the volumes of significant data in comparison to the X-ray crystallographic analysis of small viruses. Over a period of many years Michael G. Rossmann and his colleagues have often combined cryoelectron microscopy of single virus particles with X-ray structural analysis [119,120], whilst Timothy S. Baker and colleagues have tended to continue with cryo-EM alone [121].

Furthermore, because of the difficulty of obtaining complete low dose electron diffraction data from tilt series of unstained beam-sensitive crystals, the analysis of diffraction intensities for 3D reconstruction has presented considerable computational challenges. In recent years, electron diffraction data from a number of 2D and 3D crystals have been merged, a step that is necessary to achieve 3D reconstructions, such as the 3.7 Å atomic model of the tubulin dimer from zinc-induced 2D crystals by Eva Nogales and her colleagues [122]. It is likely, however, that for 3D micro-/nano-crystals more rapid progress will now be achieved, after the many years of slow progress. It is significant that viral and protein crystals were some of the first biological objects imaged by TEM [9,11,123,124], but in structural terms the hopes of these early electron microscopists still need to realised. The parallel availability of the transmission electron image and electron diffraction data from the same crystal will always be of assistance for the direct phasing of electron diffraction data for the future 3D reconstruction of proteins.

Compared to X-ray crystallography it is apparent that significant advances are still needed in instrumentation, data collection, data analysis and software development for electron diffraction technology. It remains to be seen whether electron diffraction for structure determination using small viral and protein crystals will ever be able to compete seriously with X-ray crystallography, with the current possibilities afforded by third generation synchrotron microfocus beamlines, and the future potential of X-ray free electron lasers (X-FELs).

Single particle analysis of TEM data

Although the structural analysis of single icosahedral viruses extended rapidly within the Klug group at the MRC in Cambridge [101,102] alongside the work on helical viruses [125], single particle analysis of protein molecules developed independently. Notable were the efforts of Walter Hoppe, who throughout the 1960s at the Max Planck Institute for Biochemistry moved from X-ray and electron crystallography [47,126] into the single particle TEM of analysis of ribosomes and macromolecules [127–130] using tilted specimens (work that can undoubtedly be considered, along with that of DeRosier and Klug [100], as the foundation of present day cellular and macromolecular tomography). Through the 1960s to 1990s, the Moscow-based X-ray crystallographers under Nikolay A. Kiselev [131,132] also moved into 3D electron microscopy and made interesting contributions to the subject. With the break-up of the Soviet Union and subsequent funding restriction for research, group members such as Elena V. Orlova, who possesses remarkable computing skills, moved to the West and have continued to contribute to molecular structural biology.

In the hands of Joachim Frank [133–135], Michael Radermacher [136,137], Marin van Heel [99,138,139] and their colleagues, a number of approaches for digital single particle analysis and 3D reconstruction have emerged; these and others were developed as program packages that have progressed through to the present day (see below, for Software Development). The same can be said for the more recent development of 3D tomographic reconstruction of *in situ* subcellular organelles, cytoskeletal fibrils and macromolecular complexes; Ulf Skoglund and his colleagues have pioneered this field [140].

The crucial factor for 2D crystal, single particle and tomographic structural analysis of symmetrical and asymmetrical particles has always been to recover the necessary information from TEM projection images to generate a 3D reconstruction. This problem was approached initially using negatively stained particles, in the knowledge that there may be only a limited or selective range of particle orientations available from carbon-adsorbed particles (zero tilt specimens), overcome in random conical tilt by utilizing images of the same region in two specimen tilt positions. With the advent of unstained cryo-specimens, a more complete range of freely oriented particles was achieved, but even here surface tension forces were found to impose a restricted or selective orientation in some cases. Despite its limitations, the single particle angular reconstitution approach has proved to be increasingly successful for the 3D reconstruction of many macromolecules and macromolecular complexes, to near-atomic resolution.

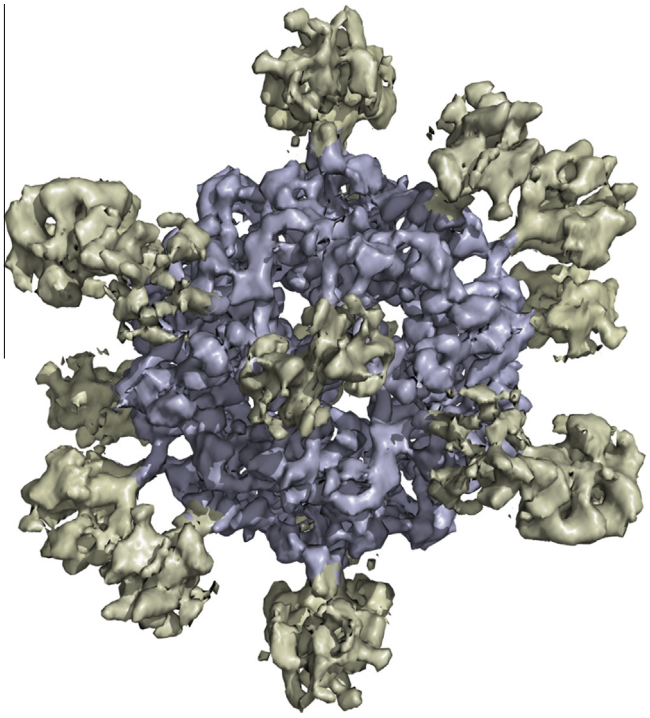

Fig. 16. A 3D reconstruction from unstained single particle cryo-electron microscopy in IMAGIC of the icosahedral *Bacillus subtilis* RsbR:RsbS stressosome complex at 6.5 Å. The diameter of the complex is ~150 Å, mass ~1 MDa. From Marles-Wright et al. (2008) [141] with the permission of the American Association for the Advancement of Science.

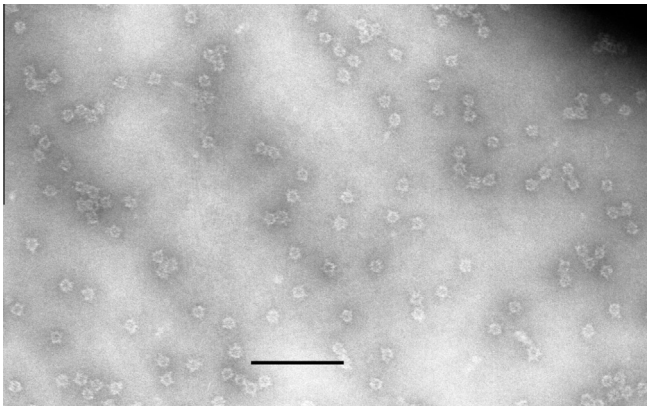

Fig. 17. The *Bacillus subtilis* RsbR:RsbT:RsbT stressosome complex, negatively stained with 5% ammonium molybdate 0.1% trehalose (equivalent to Fig. 3c in Chen et al., 2003) [142]. Note the wide range of particle orientations. The scale bar indicates 100 nm.

A representative example of the increasing success of single particle cryo-electron microscopy is of the ~1.8 MDa RsbR:RsbS stressosome complex from *Bacillus subtilis* [141], shown at 6.5 Å resolution in Fig. 16, with comparison to an unprocessed negatively stained image (Fig. 17) [142]. The attachment sites of the ternary RsbT component to the stressosome core, as a signal integration and transduction hub, were defined by this study [141].

Software development

The historical progression from optical diffraction, Fourier transform to digital image analysis and Fourier synthesis of TEM images was dependent on software development through the 1960s. The skills of R. Anthony Crowther, Richard Henderson and others led to the production of the first software package, termed the MRC image processing programs [143]. The computer literacy of the scientists involved undoubtedly facilitated progress in this area, which then spread slowly to other laboratories. Regrettably, not all electron microscopists possess such skills. The progressive availability of several other software packages for 2D and 3D image processing and molecular reconstruction from single particles (e.g., Semper, IMAGIC, EMAN, MOLREP, SPIDER and WEB, SUPRIM, XMIPP) [144–149] pushed forward image reconstruction for negatively stained and unstained frozen-hydrated macromolecules, fibrils and viruses. Independently, the user friendly crystallographic image processing program CRISP was developed by Sven Hovmöller [150] (more specifically for atomic resolution EM images of mineral samples but also with macromolecular application). Crystallographic analysis has also been incorporated into several software packages, and the combination of independent approaches – known as 'integrative structural biology' is likely to be of ever increasing significance in the future [117]. The multitude of software packages now available indicates the important

contribution made by those with advanced programming skills to the continuing development of TEM image processing and macromolecular 3D reconstruction; the *Journal of Structural Biology* published an extremely useful special issue, entitled Advances in Computational Image Processing for Microscopy (Vol. 116, No. 1, Jan./Feb. 1996) and a number of web sites are available for individual programs and lists of program packages, e.g.

http://www2.mrc-lmb.cam.ac.uk/research/locally-developed-software/image-processing-software/
http://en.wikibooks.org/wiki/Software_Tools_For_Molecular_Microscopy/General_packages
http://franklab.cpmc.columbia.edu/franklab/research/software
http://em-outreach.ucsd.edu/community-codes/MicroscopySoftware.html
http://www.c-cina.unibas.ch/links/image-processing

Molecular docking

Where the X-ray structure of a macromolecule, subunit or fragment is known, or a computer generated structure from the known amino acid sequence has been modelled [151], the possibility of fitting or docking the structure within the EM envelope has been increasingly exploited. This docking was initially performed manually, but automated docking and modelling has increasingly become the norm using procedures that are akin to molecular replacement in X-ray crystallography, to enable interaction sites and dynamic polypeptide chain movements to be defined more accurately. An example shown in Fig. 18 is the docking of the 1.7 Å X-ray structure of human erythrocyte peroxiredoxin II (PDB code 1QMV) into the negatively-stained TEM envelope [82].

Some historical examples of progress in structural biology

Numerous biomolecular examples could be mentioned that demonstrate the progressive improvement of macromolecular TEM data over the past 70 years. Several structures have been studied to progressively higher resolution. Single particle imaging of ribosomes has received constant attention since the early days of negative staining [79], immunolabelling and unstained cryo-imaging, but ultimately the asymmetric ribosome structure was solved at higher resolution by X-ray crystallography rather than by TEM [152]. Nevertheless, cryo-TEM of various ribosomes still continues to the present day. Other TEM examples such as the enzyme catalase likewise show a progression to X-ray crystallography, as do small viruses. However, the study of other materials, such as collagen, myofibrils and helical viruses (in particular TMV) [153] and several helical fibrillar proteins remain strongly in the sphere of high resolution cryo-TEM, along with a number of high molecular mass macromolecular assemblies and viruses that have proved difficult to crystallize.

The collagen molecule (an ancient protein in evolutionary terms and the predominant protein in the animal body) and the assembly of collagen fibrils is an interesting example, as this protein was studied greatly in the early days of both X-ray crystallography and TEM [154], with much progress coming from metal shadowing [16–18] and negative staining studies [155]. Nevertheless, the topic has proved somewhat intractable, partly due to the complexity of the ~300 nm-long triple helical molecule and the still incompletely understood ionic-dependency of its spontaneous assembly into D-banded and other fibrillar forms. Even today, much remains uncertain but it is certain that TEM will continue to contribute to the understanding of collagen's structure, as it has for a number of other fibrillar proteins.

TEM structural data on the molluscan hemocyanins (and indeed the hemocyanins from the arthropods) presents an even better example of the progress from the 1940s through to the present day, with a sizeable literature available. Space does not permit a thorough review of this topic, which extends from the work of von Ardenne [4] in the hands of many investigators using metal shadowing [58] and negative staining [156] through to the more recent study of frozen-hydrated hemocyanin molecules [157]. A significant advance was the production of the first molluscan hemocyain 3D reconstruction by Mellema and Klug in 1971 [106]. From negatively stained images, their low resolution 3D reconstruction revealed many salient features of the molecule. Hemocyanins from a large number of molluscan species together with their dissociation and reassociation products have also been studied, with Ernst van Bruggen and his group making a major contribution through the 1960s to 1980s. Together with Kensal E. van Holde [158], others, such as Jean N. Lamy and Jürgen Markl and their colleagues and collaborators, joined this field of research through the 1990s to 2000s.

The studies on Keyhole limpet (*Megathura crenulata*) hemocyanin (KLH) (Fig. 19) [159], a molecule that possesses remarkable immunostimulatory properties (available commercially as the clinical oncology product Immucothel®, Biosyn Arzneimittel GmbH), have been prominent [160]. The Markl group has concentrated on both the structure and molecular genetics of KLH, alongside hemocyanins from other gastropod species, such as the European abalone *Haliotis tuberculatis* and the cephalopod mollusc *Nautilus pompilius*. They published the 3D structure of KLH from cryo-electron microscopy in a progressive manner over a number of years. This led to a 9 Å structure of the KLH didecamer in 2009 [161], as shown in Fig. 20, only to be surpassed by a higher resolution (4.5 Å) structure of *Haliotis diversicolor* hemocyanin by Zhang and colleagues in 2013 [162]. Although many researchers have struggled to produce diffracting 3D crystals from molluscan hemocyanins for X-ray crystallography, it can be anticipated that before long this will be achieved, as it has for some arthropod hemocyanins, to yield a yet higher resolution 3D structure.

Final comments

This historical overview provides a personal insight into the progress in the field of molecular structural biology and emphasises the almost indispensible contribution that the TEM has made. Since the early biological TEM studies of Helmut and Ernst Ruska, Manfred von Ardenne [2–4,163] and others such as Robley C. Williams and Ralph W.G. Wyckoff [5,8,9], progress has been tremendous. Indeed, worthy of further emphasis is the outstanding contribution of Wyckoff, working in the Laboratory of Physical Biology at the NIH in Bethesda, immediately apparent from his numerous journal publications and the bacteriological, virological and macromolecular content of his 1949 book *Electron Microscopy: Technique and Applications*, in which he achieved an impressive academic standard, with thorough referencing to many early TEM studies. Similar comments can be made about the 1953 and later editions of the book *Introduction to Electron Microscopy* by Cecil E. Hall, working at MIT. Whilst being particularly strong on the more instrumental aspects, the examples given from applied electron microscopy lean strongly towards biomolecular applications.

Over the past 80 years since the invention of the transmission electron microscope, it has become clearly apparent that an increasing number of laboratories around the world are now contributing to macromolecular structure studies with this electron optical instrument. This has been assisted by the availability of excellent quality TEMs from several manufacturers, many of which

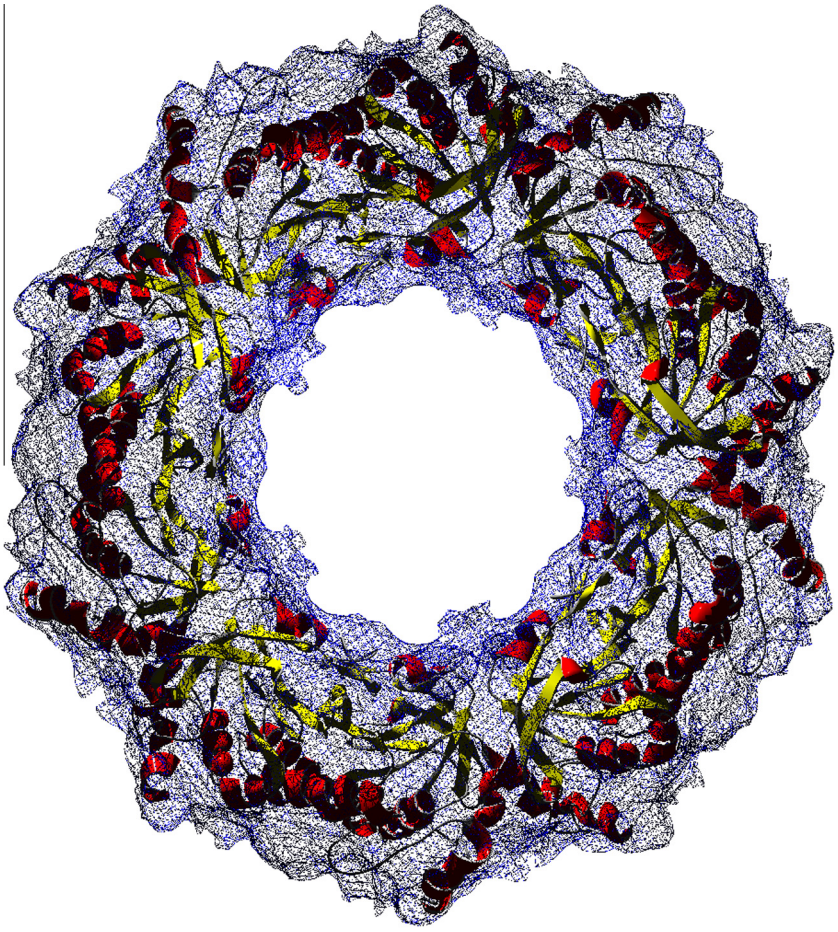

Fig. 18. Human erythrocyte peroxiredoxin-II with docking of the 1.7 X-ray ribbon model onto the scaled 19 Å TEM 3-D reconstruction electron density. This was performed by molecular replacement using MOLREP. The figure was generated using MOLSCRIPT. Modified from Fig. 10 of Harris et al. (2001) [82] with the permission of Elsevier.

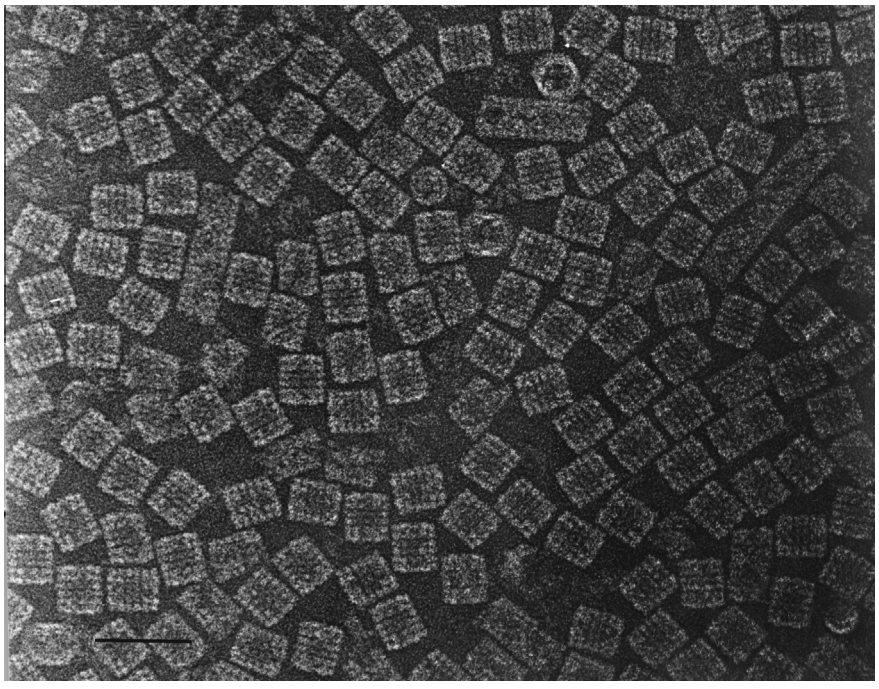

Fig. 19. Reassociated KLH1 didecamers negatively stained with 5% ammonium molybdate containing 1% trehalose (pH 7.0) using a holey carbon support film, and imaged at low electron dose with liquid nitrogen cooling of the specimen. This image is typical of molluscan hemocyanin didecamers revealed by negative staining of hemocyanin from many species. From Harris et al. (1998) [159] with the permission of Elsevier.

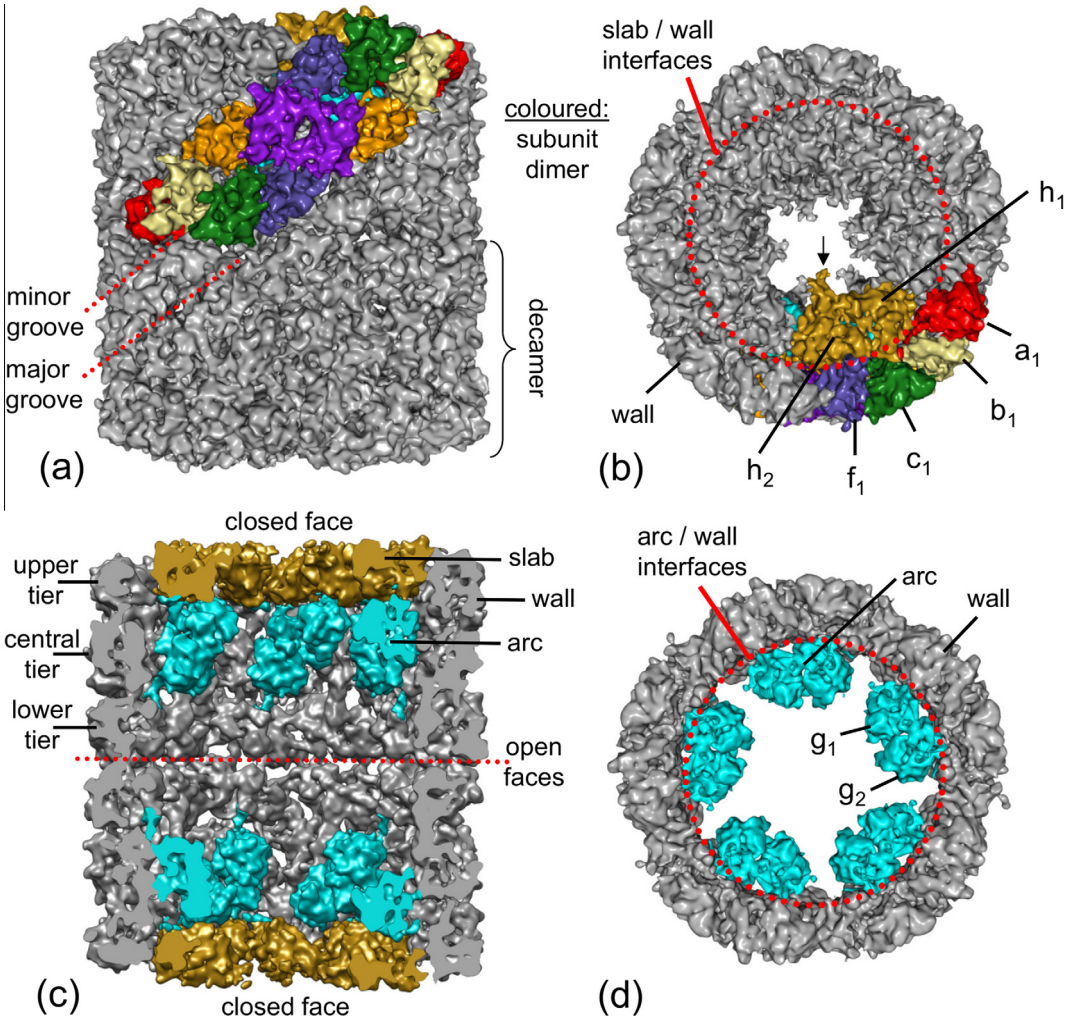

Fig. 20. 9-Å cryoEM structure of the KLH1didecamer. (a) Side view, (b) top view, and (c) cut-open view of the didecamer. (d) Top view of one extracted decamer, with the slab pentamer removed. The eight FUs (a–g) are indicated (KLH1-a, red; KLH1-b, yellow; KLH1-c, green; KLH1-d, orange; KLH1-e, purple; KLH1-f, blue; KLH1-g, cyan; KLH1-h, gold). In (a), note the shape of the subunit dimer (colored). Also note the prominent protrusions from the collar narrowing the cylinder lumen (arrow in (b)), which are interpreted as N-linked glycans. From Christos Gatsogiannis and Jürgen Markl (2009) [161], with the permission of Elsevier (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this paper.).

continue to perform well beyond their initially expected life-span, along with an increasing number of cryo-instruments. Indeed, the impact of Cryo-EM cannot be overstated. Stemming primarily from the 1980s work of Jacques Dubochet and his colleagues at the EMBL in Heidelberg, this technical advancement can be seen as the most significant innovation in electron optical molecular structural biology. It is pertinent to quote from the 1983 article by Lepault et al. [164]: "*By changing the concentration and composition of the aqueous medium the contrast of particles in a vitreous film can be controlled and any state of negative, positive or zero contrast may be obtained*". This seminal statement succinctly defines the electron imaging of both unstained and negatively stained vitrified biological particulates.

Over the years there have been recurring themes, such as the study of TMV and other viruses, ribosomes, ferritin, fibrillar proteins such as fibrin, collagen, myosin, actin, RecA, intermediate filaments and microtubules, together with numerous oligomeric enzymes and complexes, including the gastropod and arthropod hemocyanins, from which progress can be immediately appreciated. One can but admire the efforts of so many of the earlier biologists who established the base from which we continue TEM work today. The increasing availability of software packages for TEM image analysis and 3D macromolecular reconstruction advanced alongside the steady, if slow, improvement of specimen preparation techniques upon which the generation of new and improved data depends. The establishment of the EMDataBank (a unified data source for 3DEM) in 2002 provided an open access source for the whole 3DEM community, in parallel with the 3D crystallographic Protein Data Bank (PDB). A strong link with cellular ultrastructure remains, increasingly through cryo-electron tomography, which is contributing significantly at both the subcellular and macromolecular level.

It is undoubtedly encouraging for the early workers in this field that so many different laboratories around the world, increasingly in Asia, are now contributing to TEM structural studies on a vast range of macromolecules, macromolecular complexes and viruses, which will continue to contribute to both structural and associated functional knowledge. Historically speaking, we have travelled far since the statement by J. D. Bernal (1968): "*A characteristic feature of all recent electron microscope photographs is the essentially topologically definite structures which are apparently fluid in form and without rectilinear features*", in the light of present-day knowledge and the likely future prospect of routinely achieving atomic resolution in TEM 3D macromolecular reconstructions! Interesting historical video recordings of Aaron Klug and other pioneers can be found on the "YouTube" and "Web of stories" web sites.

It is a significant observation that the students and successors of so many of the pioneers of macromolecular electron microscopy have themselves established successful careers, spread across numerous aspects of this field of study, leading to the high resolution 3D structural data that is being produced today. Progress was firmly predicted in 1974 [165] and the field was elegantly and optimistically reviewed in 2010 by R. Anthony Crowther [166], with emphasis on the impressive long-term contribution from the MRC Laboratory of Molecular Biology in Cambridge. The increasing integration of X-ray crystallographic and NMR data with that from TEM imaging and diffraction has been a historical and on-going strength. TEM data from the numerous macromolecular complexes, fibrils and viruses that cannot yet be crystallized will undoubtedly continue to be a source of amazement for many years to come, as enthusiastically summarized by Werner Kühlbrant [167].

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References

[1] L. Marton, Bull. Acad. Roy. Med. Belg. 20 (1934) 439–446.
[2] G. Kauseche, E. Pfankuch, H. Ruska, Naturwissenschaften 27 (1939) 292–299.
[3] E. Husemann, H. Ruska, Naturwissenschaften 28 (1940) 534.
[4] M. von Ardenne, Naturwissenschaften 8 (1940) 112–127.
[5] R.C. Williams, R.W.G. Wyckoff, Nature 156 (1945) 68–70.
[6] W.T. Astbury, S.V. Perry, R. Reed, L.C. Spark, Biochim. Biophys. Acta 1 (1947) 379–392.
[7] W. van Iterson, Biochim. Biophys. Acta 1 (1947) 527–548.
[8] R.W.G. Wyckoff, Biochim. Biophys. Acta 1 (1947) 143–146.
[9] R.W.G. Wyckoff, Acta Crystallogr. 1 (1948) 292–294.
[10] L. Algera, J.J. Beijer, W. van Iterson, W.K.H. Karstens, T.H. Thung, Biochim. Biophys. Acta 1 (1947) 517–526.
[11] R. Markham, K.M. Smith, R.W.G. Wyckoff, Nature 159 (1947) 547.
[12] A.S. MacFarlane, Br. Med. J. 3 (1949) 1247–1250.
[13] K. Muhlethaler, Biochim. Biophys. Acta 3 (1949) 15–25.
[14] S.E. Luria, M. Delbrück, T.F. Anderson, J. Bacteriol. 46 (1943) 57–77.
[15] W.M. Stanley, T.F. Anderson, J. Biol. Chem. 146 (1942) 25–30.
[16] J. Gross, F.O. Schmitt, J. Exp. Med. 88 (1948) 555–568.
[17] J.H. Highberger, J. Gross, F.O. Schmitt, Proc. Natl. Acad. Sci. U.S.A. 37 (1951) 286–292.
[18] J. Gross, J.H. Highberger, F.O. Schmitt, Proc. Natl. Acad. Sci. U.S.A. 40 (1954) 679–688.
[19] J. Kuo (Ed.), Electron Microscopy Methods and Protocols, third ed., Humana Press, 2014.
[20] R. Henderson, Arch. Biochem. Biophys. XX (2015) (XX–XX).
[21] R.W. Horne, Virus Structure, Academic Press, London, 1974.
[22] J.R. Harris, R.W. Horne (Eds.), Electron microscopy of proteins, Virus Structure, Vol. 5, Academic Press, London, 1986.
[23] M.V. Nermut, A.C. Steven (Eds.), Animal Virus Structure, Elsevier, Amsterdam, 1987.
[24] W. Chiu, J. Johnson (Eds.), Virus Structure, Advances in Protein Chemistry, Vol. 64, Elsevier Science, San Diego, 2003.
[25] W. Chiu, R.M. Burnett, R.L. Garcea, Structural Biology of Viruses, Oxford University Press, 1997.
[26] D.E. Bradley, Bacteriol. Rev. 34 (1967) 230–314.
[27] R.C. Williams, Adv. Virus Res. 2 (1954) 183–239.
[28] A. Engel, Ultramicroscopy 37 (1978) 273–281.
[29] G. Palade, J. Cell Biol. 50 (1971) 5D–19D.
[30] T. Shirahama, A.S. Cohen, J. Cell Biol. 33 (1967) 679–708.
[31] U. Aebi, W.E. Fowler, P. Rew, T.T. Sun, J. Cell Biol. 97 (1983) 1131–1143.
[32] A.N.J. Heyn, J. Cell Biol. 29 (1966) 181–191.
[33] H. Fernández-Morán, Ann. N. Y. Acad. Sci. 85 (1960) 689–713.
[34] K.A. Taylor, R.M. Glaeser, Science 186 (1974) 1036–1037.
[35] K.A. Taylor, R.M. Glaeser, J. Ultrastruct. Res. 55 (1976) 448–456.
[36] W. Chiu, High resolution electron microscopy of unstained, hydrated protein crystals, in: J.R. Harris (Ed.), Electron Microscopy of Proteins, Vol. 2, Academic Press, London, 1981, pp. 233–259.
[37] W. Chiu, Ann. Rev. Biophys. Chem. 15 (1986) 237–257.
[38] K.A. Taylor, R.M. Glaeser, J. Struct. Biol. 163 (2008) 214–223.
[39] J. Dubochet, M. Adrian, J.-H. Chang, J.-C. Homo, J. Lepault, A.W. McDowall, P. Schultz, Q. Rev. Biophys. 21 (1988) 129–228.
[40] J. Dubochet, J. Microsc. 245 (2012) 221–224.
[41] B. Roche, M. Bessis, J.-P. Thiery, Biochim. Biophys. Acta 41 (1960) 182–184.
[42] S. Fitton Jackson, Proc. R. Soc. London B 144 (1956) 556–572.
[43] J. Hanson, H.E. Huxley, Nature 172 (1953) 530–532.
[44] H.E. Huxley, J. Hanson, Nature 173 (1954) 973–976.
[45] H.E. Huxley, Biochim. Biophys. Acta 12 (1953) 375–394.
[46] R.H. Lange, Int. Rev. Cytol. 97 (1985) 133–181.
[47] W. Hoppe, R. Langer, G. Knesch, C. Poppe, Naturwissenschaften 55 (1968) 333–336.
[48] C.E. Hall, Proc. Natl. Acad. Sci. U.S.A. 42 (1956) 801–806.
[49] C.E. Hall, P. Doty, J. Am. Chem. Soc. 80 (1958) 1269–1274.
[50] R.W.G. Wyckoff, Adv. Protein Chem. 6 (1951) 1–33.
[51] A. Kleinschmidt, R.K. Zahn, Z. Naturforsch B 14 (1959) 770–779.
[52] H. Gross, T. Müller, I. Wildhaber, H. Winkler, Ultramicroscopy 16 (1985) 287–304.
[53] H.S. Slayter, Electron microscopy of glycoproteins, in: J.R. Harris (Ed.), Electron Microscopy of Proteins, Vol. 1, Academic Press, London, 1981, pp. 198–254.
[54] G.C. Ruben, Micron 29 (1998) 359–396.
[55] N. Braun, W. Meining, U. Hars, M. Fischer, R. Ladenstein, R. Huber, A. Bacher, S. Weinkauf, L. Bachman, J. Mol. Biol. 32 (2002) 341–353.
[56] J.R. Harris, The structure of the red blood cell membrane using the electron microscope (Ph.D. thesis), University of Edinburgh, Fig. 30, 1968.
[57] J.R. Harris, W. Gebauer, S.M. Söhngen, M.V. Nermut, J. Markl, Micron 28 (1997) 43–56.
[58] J.E. Heuser, J. Mol. Biol. 169 (1983) 155–195.
[59] A.E. Blaurock, W. Stoeckenius, D. Oesterhelt, G.L. Scherphof, J. Cell Biol. 71 (1976) 1–22.
[60] G.M. Hendricks, Metal shadowing for electron microscopy, in: J. Kuo (Ed.), Electron Microscopy: Methods and Protocols, Humana Press, 2014, pp. 73–93.
[61] H.E. Huxley, in: First Europ. Conf. on Electron Microscopy, Stockholm, 1956, pp. 260–261.
[62] C.E. Hall, J. Biophys. Biochem. Cytol. 1 (1955) 1–15.
[63] S. Brenner, R.W. Horne, Biochim. Biophys. Acta 34 (1959) 103–110.
[64] R.W. Horne, P. Wildy, Adv. Virus Res. 10 (1964) 102–170.
[65] R. Valentine, Nature 184 (1959) 1838–1841.
[66] H.E. Huxley, G. Zubay, J. Mol. Biol. 2 (1960) 189–196.
[67] D.E. Bradley, J. Gen. Microbiol. 29 (1962) 503–516.
[68] E.L. Benedetti, P. Emmelot, J. Cell Biol. 26 (1965) 299–305.
[69] J.R. Harris, Negative Staining and Cryoelectron Microscopy. RMS Handbook No. 53, BIOS Scientific Publishers Ltd, Oxford, 1997.
[70] J.R. Harris, S. DeCarlo, Negative staining and cryo-negative staining: applications in biology and medicine, in: J. Kuo (Ed.), Electron Microscopy Methods and Protocols, Humana Press, 2014, pp. 215–258.
[71] T.F. Anderson, Negative staining and its use in the study of viruses and their serological reactions, in: R.J.C. Harris (Ed.), The Interpretation of Ultrastructure, Academic press, London, 1962, pp. 251–262.
[72] A.J. Dalton, F. Haguenau, Ultrastructure of Animal Viruses and Bacteriophages, Academic Press Inc, New York and London, 1973.
[73] R.C. Valentine, R.W. Horne, An assessment of negative staining techniques for revealing ultrastructure, in: R.J.C. Harris (Ed.), The Interpretation of Ultrastructure, Academic press, London, 1962, pp. 263–278.
[74] R.H. Haschemeyer, E. de Harven, Ann. Rev. Biochem. 43 (1974) 279–301.
[75] R.C. Valentine, B.M. Shapiro, E.R. Stadman, Biochemistry 7 (1968) 2143–2152.
[76] R.W. Horne, I. Pasquali-Ronchetti, J. Ultrastruct. Res. 47 (1974) 361–383.
[77] B. Wells, R.W. Horne, P.J. Shaw, Micron 12 (1981) 37–45.
[78] J.A. Lake, Meth. Enzymol. 61 (1979) 250–257.
[79] H.E. Huxley, G. Zubay, J. Mol. Biol. 2 (1960) 10–18.
[80] J.R. Harris, D. Scheffler, Micron 33 (2002) 461–480.
[81] J.R. Harris, Micron 39 (2008) 168–176.
[82] J.R. Harris, E. Schröder, M.N. Isupov, D. Scheffler, P. Kristensen, J.A. Littlechild, A.A. Vagin, U. Meissner, Biochim. Biophys. Acta 1547 (2001) 221–234.
[83] J.R. Harris, J. Mol. Biol. 46 (1969) 329–335.
[84] L. Zhang, J. Song, Y. Newhouse, S. Zhang, K.H. Weisgraber, G. Ren, J. Lipid Res. 51 (2010) 1228–1236.
[85] M. Adrian, J. Dubochet, S. Fuller, J.R. Harris, Micron 29 (1998) 145–160.
[86] M. Adrian, J. Dubochet, J. Lepault, A.W. McDowall, Nature 308 (1984) 32–36.
[87] U. Meissner, E. Schröder, D. Scheffler, A.G. Martin, J.R. Harris, Micron 38 (2007) 29–39.
[88] S. De Carlo, H. Stark, Meth. Enzymol. 418 (2010) 127–145.
[89] J.R. Harris, M. Adrian, in: M.A.N. Hajibagheri (Ed.), Meth. Meth. Mol. Biol., Vol. 117, Humana Press, 1999, pp. 31–48.
[90] M. Stewart, in: J.R. Harrris (Ed.), Electron Microscopy in Biology, IRL Press, 1991, pp. 229–242.
[91] M.J. Dobro, L.A. Melanson, G.J. Jensen, A.W. McDowall, Meth. Enzymol. 481 (2010) 63–82.
[92] D.W. Taylor, D.F. Kelly, A. Cheng, K.A. Taylor, J. Struct. Biol. 160 (2007) 305–312.
[93] J.R. Harris, Methods 10 (1996) 234–246.
[94] L. Kahn, D.A. Winkelmann, J.A. Lake, J. Mol. Biol. 145 (1981) 193–214.
[95] J.C. Mitchell, J.R. Harris, J. Malo, J. Bath, A.J. Turberfield, J. Am. Chem. Soc. 126 (2004) 16342–16343.
[96] J.F. Hainfeld, W. Liu, C.M.R. Halsey, P. Freimuth, R.D. Powell, J. Struct. Biol. 127 (1999) 185–198.
[97] A. Klug, J.T. Finch, J. Mol. Biol. 11 (1965) 403–423.
[98] A. Klug, J.T. Finch, J. Mol. Biol. 31 (1968) 1–12.

[99] M. van Heel, B. Gowen, R. Matadeen, E.V. Orlova, R. Finn, T. Pape, D. Cohen, H. Stark, R. Schmidt, M. Schatz, A. Patwardhan, Q. Rev. Biophys. 33 (2000) 307–369.
[100] D.J. De Rosier, A. Klug, Nature 217 (1968) 130–134.
[101] R.A. Crowther, Philos. Trans. R. Soc. London B 261 (1971) 221–230.
[102] R.A. Crowther, L.A. Amos, J.T. Finch, D.J. De Rosier, A. Klug, Nature 226 (1970) 421–425.
[103] R. Henderson, P.N.T. Unwin, Nature 257 (1975) 28–32.
[104] P.N.T. Unwin, R. Henderson, J. Mol. Biol. 94 (1975) 425–440.
[105] A. Klug, J.E. Berger, J. Mol. Biol. 10 (1964) 565–569.
[106] J.E. Mellema, A. Klug, Nature 239 (1972) 146–150.
[107] R.W. Horne, R. Markham, Applications of optical diffraction and image reconstruction techniques to electron micrographs, in: A.M. Glauert (Ed.), Practical Methods in Electron Microscopy, vol. 1, North-Holland Publishing Co., Amsterdam, 1973, pp. 327–444.
[108] V.R. Matricardi, R.C. Moretz, D.F. Parsons, Science 177 (1972) 268–269.
[109] M.B. Tsuprun, E.V. Orlova, S.S. Terzyan, R. Kleine, N.A. Kiselev, Ultramicroscopy 7 (1981) 131–138.
[110] P.N.T. Unwin, J. Mol. Biol. 98 (1975) 235–242.
[111] D.L. Dorset, D.F. Parsons, Acta Crystallogr. A 31 (1975) 210–215.
[112] N. Chanzy, J.-M. Franc, D. Herbage, Biochem. J. 153 (1976) 139–140.
[113] W. Baumeister, W. Vogel, Electron Microscopy at Molecular Dimensions, Springer-Verlag, Heidelberg, 1980.
[114] P.-L. Chiu, D.F. Kelly, T. Walz, Micron 42 (2011) 762–772.
[115] I. Schmidt-Krey, Y. Cheng (Eds.), Electron Crystallography of Soluble and Membrane Proteins, Humana Press, 2013.
[116] A. Brisson, P.N.T. Unwin, J. Cell Biol. 99 (1984) 1202–1211.
[117] D. Shi, B.L. Nannenga, M.G. Iadanza, T. Gonen, eLife 2 (2013) e01345 (http://dx.doi.org/10.7554/eLife. 01345).
[118] R.W. Horne, Optical diffraction analysis of periodically repeating biological structures, in: G.A. Meek, H.Y. Elder (Eds.), Analytical and Quantitative Methods in Microscopy, Cambridge University Press, 1977, pp. 29–53.
[119] P. Plevka, R. Perera, M.L. Yap, J. Cardosa, R.J. Kuhn, M.G. Rossmann, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) 5463–5467.
[120] M.G. Rossmann, Q. Rev. Biophys. 46 (2013) 133–180.
[121] G. Cardone, X. Yan, R.S. Sinkovits, J. Tang, T.S. Baker, J. Struct. Biol. 183 (2013) 329–341.
[122] E. Nogales, S.G. Wolf, K.H. Downing, Nature 391 (1998) 199–202.
[123] L.W. Labar, R.W.G. Wyckoff, Biochim. Biophys. Acta 25 (1957) 263–266.
[124] W. Longley, J. Mol. Biol. 30 (1967) 323–327.
[125] J. Finch, A. Klug, J. Mol. Biol. 87 (1974) 633–640.
[126] W. Hoppe, Acta Crystallogr. A26 (1970) (441–426).
[127] W. Hoppe, Naturwissenschaften 61 (1974) 239–249.
[128] W. Hoppe, B. Grill, Ultramicroscopy 2 (1977) 153–168.
[129] W. Hoppe, H. Tietz, Ultramicroscopy 20 (1986) 217–238.
[130] W. Hoppe, H. Oettl, H.R. Tietz, J. Mol. Biol. 192 (1986) 291–322.
[131] N.A. Kiselev, V.Y. Stel'mashchuk, H. Lutsh, F. Noll, J. Mol. Biol. 126 (1978) 109–115.
[132] N.A. Kiselev, M.B. Sherman, V.L. Tsuprun, Electron Microsc. Rev. 3 (1990) 43–72.
[133] J. Frank, W. Goldfarb, D. Eisenberg, T.S. Baker, Ultramicroscopy 3 (1978) 283–290.
[134] J. Frank, Electron Microsc. Rev. 2 (1989) 53–74.
[135] J. Frank, Q. Rev. Biophys. 23 (1990) 281–329.
[136] M. Radermacher, J. Electron Microsc. Technol. 9 (1988) 359–394.
[137] M. Radermacher, Ultramicroscopy 53 (1994) 121–136.
[138] M. van Heel, J. Frank, Ultramicroscopy 6 (1981) 187–194.
[139] M. van Heel, Ultramicroscopy 21 (1987) 111–124.
[140] U. Skoglund, K. Andersson, B. Strandberg, B. Daneholt, Nature 319 (1986) 560–564.
[141] J. Marles-Wright, T. Grant, O. Delumeau, G. Van Duinen, S.J. Firbank, P.J. Lewis, J.W. Murray, M.B. Quin, P.R. Race, A. Rohou, W. Tichelaar, M. Van Heel, R.J. Lewis, Science 322 (2008) 92–96.
[142] C.C. Chen, R.J. Lewis, J.R. Harris, M.D. Yudkin, O. Delumeau, Mol. Microbiol. 49 (2003) 1657–1669.
[143] R.A. Crowther, R. Henderson, J.M. Smith, J. Struct. Biol. 116 (1996) 123–130.
[144] W.O. Saxton, J. Struct. Biol. 116 (1996) 230–236.
[145] M. Van Heel, G. Harauz, E.V. Orlova, R. Schmidt, M. Schatz, J. Struct. Biol. 116 (1996) 17–24.
[146] S.J. Ludtke, P.R. Baldwin, W. Chiu, J. Struct. Biol. 116 (1999) 82–97.
[147] J. Frank, M. Radermacher, P. Penczek, J. Zhu, Y. Li, M. Ladjadj, A. Leith, J. Struct. Biol. 116 (1996) 190–199.
[148] J.P. Schroeter, J.-P. Bretaudiere, J. Struct. Biol. 116 (1996) 131–137.
[149] A. Vagin, A. Teplyakov, Acta Crystallogr. D Biol. Crystallogr. 66 (2010) 22–25.
[150] S. Hovmöller, Ultramicroscopy 41 (1992) 121–135.
[151] K.J. Neil, R.A. Ridsdale, B. Rutherford, L. Taylor, D.E. Larson, M. Glibetic, L.I. Rothblum, G. Harauz, Nucleic Acids Res. 24 (1996) 1472–1480.
[152] J.H. Cate, M.M. Yusupov, G.Zh. Yusupova, T.N. Earnest, H.F. Noller, Science 285 (1999) 2095–2104.
[153] C. Sachse, J.Z. Chen, P.-D. Coureux, M.E. Stroupe, M. Fändrich, N. Grigorieff, J. Mol. Biol. 371 (2007) 812–835.
[154] A. Serafini-Fracassini, in: J.R. Harris (Ed.), Electron Microscopy of Proteins, Vol. 2, Academic Press, London, 1982, pp. 195–231.
[155] J.R. Harris, A. Soliakov, R.J. Lewis, Micron 49 (2013) 60–68.
[156] H. Fernández-Morán, E.F.J. van Bruggen, M. Ohtsuki, J. Mol. Biol. 16 (1966) 191–207.
[157] O. Lambert, N. Boisset, J.-C. Taveau, J.N. Lamy, J. Mol. Biol. 244 (1994) 640–647.
[158] K.E. van Holde, K. Miller, E. Schabtach, L. Libertini, J. Mol. Biol. 217 (1991) 307–321.
[159] J.R. Harris, W. Gebauer, M. Adrian, J. Markl, Micron 29 (1998) 329–339.
[160] J.R. Harris, J. Markl, Micron 30 (1999) 597–623.
[161] C. Gatsogiannis, J. Markl, J. Mol. Biol. 385 (2009) 963–983.
[162] Q. Zhang, X. Dai, Y. Cong, J. Zhang, D.-H. Chen, M.T. Dougherty, J. Wang, S.J. Ludke, M.F. Schmid, W. Chiu, Structure 21 (2013) 604–613.
[163] H. Ruska, B. Von Borries, E. Ruska, Arch. Virusforch. 1 (1939) 155–169.
[164] J. Lepault, F.P. Booy, J. Dubochet, J. Microsc. 129 (1983) 89–102.
[165] M. Beer, J. Frank, K.-J. Hanszen, E. Kellenberger, R.C. Williams, Q. Rev. Biophys. 7 (1974) 211–238.
[166] R.A. Crowther, Adv. Protein Chem. Struct. Biol. 81 (2010) 1–32.
[167] W. Kuhlbrant, Science 343 (2014) 1443–1444.